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Solution structure and NMR characterization of the binding to methylated histone tails of the plant homeodomain finger of the tumour suppressor ING4

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Abstract **Plant homeodomain (PHD) fingers are frequently present in proteins involved in chromatin remodelling, and some of them bind to histones. The family of proteins inhibitors of growth (ING) contains a PHD finger that bind to histone-3 trimethylated at lysine 4, and those of ING1 and ING2 also act as nuclear phosphoinositide receptors. We have determined the structure of ING4 PHD, and characterised its binding to phosphoinositides and histone methylated tails. In contrast to ING2, ING4 is not a phosphoinositide receptor and binds with similar affinity to the different methylation states of histone-3 at lysine 4.**

Keywords: ING4; PHD; Histone binding; Phosphoinositide binding; Tumour suppressor; Chromatin remodelling

1. Introduction

The inhibitor of growth (ING) family of tumour suppressors [1] consists of five homologous proteins which form stable complexes with other proteins involved in the regulation of chromatin acetylation [2]. N-terminal histone tail modification is a key mechanism of regulation of chromatin structure, and the pattern of histone modification around a gene affects its transcription [3]. Histone acetylation and methylation at lysines are the most common modifications, and are recognised by specific protein domains [4]. ING proteins contain a conserved C-terminal plant homeodomain (PHD) finger [5], also present in many nuclear proteins involved in gene expression regulation and chromatin remodelling [6]. The PHD of p300 and ACF1 bind to nucleosome histones, and since both proteins contain also a bromodomain, which recognise acetylated lysines, they could form an integrated nucleosome recognition module [7,8]. PHD fingers could bind preferentially to methylated ones, as do chromodomains. This has been confirmed by the recent report that the PHD of ING proteins [9,10] and the PHD of NURF [11,12] bind to histone-3 trimethylated at lysine 4 (H3K4me3).

The PHD fingers of ING1 and ING2 are also nuclear receptors of phosphoinositides [13]. These phospholipids recruit proteins to the vicinity of the membranes regulating cell survival, growth and proliferation. Their interaction with the PHD could regulate the nuclear response to cellular stress [14].

Here, we describe the solution structure of the PHD finger of ING4 and the characterization of its binding to phosphoinositides and histone methylated tails. The results are compared with those reported for ING2 and their functional implications are discussed.

2. Materials and methods

2.1. Protein expression and purification

The PHD finger of ING4 (residues 188–249 with an extra methionine at the N-terminus) was subcloned into the expression vector pET11d from a plasmid harbouring the synthetic gene of ING4 with codons optimized for expression in *Escherichia coli* (Entelechon GmbH). PHD mutants were constructed with QuickChange (Stratagene).

Proteins were over-expressed in *E. coli* BL21(DE3) cells grown at 37 °C in rich medium supplemented with 50 μM $ZnCl_2$ and harvested after 4 h of induction with 0.5 mM isopropyl-beta-D-thiogalactopiranoside. Labeled proteins were produced in minimal medium with $^{15}NH_4Cl$ and [$^{13}C_6$] glucose. After sonication and ultracentrifugation, proteins were found predominantly in the pellet, solubilised in 6 M urea and refolded by a 1:10 fold dilution into cold 20 mM Tris pH 8.0, 50 μM $ZnCl_2$. Purification by anion-exchange chromatography and gel filtration yielded proteins whose identity was confirmed by mass spectrometry. A small amount of wild-type PHD was purified directly from the soluble fraction yielding identical 1D nuclear magnetic resonance (NMR) spectrum as the refolded protein.

2.2. NMR spectroscopy and structure determination

NMR experiments were recorded on Bruker AVANCE 600 (with cryoprobe) and 700 spectrometers at 298 K in 20 mM sodium phosphate pH 6.5, 50 mM NaCl, 1 mM deuterated dithiothreitol (DTT) and 9% or 100% D_2O. Some samples also contained 0.03% NaN_3. Backbone and sidechain resonance assignment were obtained using a set of triple resonance experiments recorded on a 1.2 mM PHD sample. Chemical shifts were measured relative to internal 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) for ^1H and calculated for ^{15}N and ^{13}C [15]. Spectra were processed with XWINMR (Bruker) or NMRPipe [16] and analyzed using NMRView [17]. Distance restraints were obtained from 2D-NOESY and 3D-NOESY spectra

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Abbreviations: HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser enhancement spectroscopy; PHD, plant homeodomain; ING, inhibitor of growth; WT, wild type; DTT, dithiothreitol; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt; PIP5, D-myo-phosphatidylinositol 5-phosphate; UV, ultra violet; CSP, chemical shift perturbation

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edited in ^{13}C or ^{15}N (120 ms mixing time). Dihedral angle restraints were obtained from an HNHA spectrum and from the backbone chemical shifts using TALOS [18]. Structures were calculated with DYANA [19] and used for NOE assignment in an iterative manner. The structures were refined by energy minimization with AMBER 7.0 [20] (see Supplementary Material for the table with the structure statistics for the ensemble of the 25 refined models). The resonance assignment has been deposited with the BMRB entry 7210. The refined models have been deposited in the Protein Data Bank with the entry 2JMQ. These structures are similar to those deposited as PDB entries 1WEN and 1WEU, which contain long segments with extraneous residues at the chain termini and do not include the last four residues of the ING4 sequence.

2.3. Ligand binding

Methylated histone peptides were purchased from NeoMPS, Strasbourg, and contain an extra tyrosine residue at the C-terminus to measure peptide concentration by ultra violet (UV) absorbance. The sequences of the unmodified histone peptides are NH_2-ARTKQTARKSTGGKAY-COOH (residues 1–15 of histone-3), and NH_2-GGAKRHKVLRDNIQY-COOH (residues 14–27 of histone-4). The residues that were methylated in the different peptides are underlined. Stock peptide solutions (5–6 mM) were prepared in 20 mM sodium phosphate pH 6.5, 50 mM NaCl, and the binding was identified by the perturbation in the chemical shifts observed in ^{1}H–^{15}N-HSQC spectra of 50 μM PHD samples in the absence or presence of a 1:4 excess peptide dialysed simultaneously against the same buffer. Titrations were performed by stepwise addition of peptide stock solutions into 50 μM PHD samples and measuring the changes in the chemical shifts of W237 peak in ^{1}H–^{15}N-HSQC. Dissociation constants (K_D) were determined by data fitting (Origin, Microcal) to the equation: $\Delta\delta = (K_D + [P]+[L] - \mathrm{sqrt}((K_D + [P] + [L])^2 - 4*[P]*[L]))/(2*[P])*\Delta\delta_{max}$, where $[L]$ is the concentration of the peptide, $[P]$ is the concentration of PHD, $\Delta\delta$ is the measured chemical shift perturbation (CSP) and $\Delta\delta_{max}$ is the maximum difference in chemical shifts of the free protein and the ligand-bound protein. $\Delta\delta$ was calculated from the equation: $\Delta\delta = \mathrm{sqrt}(((\Delta\delta_H)^2 + (\Delta\delta_N/5)^2)*0.5)$, where $\Delta\delta_H$ and $\Delta\delta_N$ are the chemical shift changes in the ^{1}H and ^{15}N resonances, respectively, upon peptide addition.

Soluble PI5P was from Echelon Biosciences. Its binding was tested with ^{1}H–^{15}N HSQC spectra of 60 μM PHD in the absence or presence of a 1:10 excess of PI5P.

3. Results and discussion

The PHD finger of ING4 requires Zn^{2+} for proper folding. An interleaved finger scaffold consisting of the C4HC3 sequence motif coordinates two Zn^{2+} atoms that stabilise the loops, the antiparallel β-sheet and the one turn helix (Fig. 1), which form the typical fold of this domain [6,21]. The structure is well defined with higher variability at the chain termini, which is due to increased mobility as shown by heteronuclear ^{1}H–^{15}N NOEs (data not shown). The surface of the molecule has a region with high density of positive charge (Fig. 1C) with a large contribution from the last four residues at the C-terminus (RKKK). This region could be involved in the binding to phosphoinositides, in a similar way as a positively charge region present in ING1 and ING2 after the PHD sequences (see Supplementary Material) is necessary for phosphoinositide binding [13]. Similar findings have been reported for the PHD of Pf1 [22]. However, ING4 PHD finger does not bind to a panel of different phosphoinositides, or does so with an extremely low affinity, undetectable in solution by NMR (Fig. 3), and barely detectable in an overexposed dot blot with immobilized phosphoinositides (see Supplementary Material). Phosphoinositide binding is not a property of all PHD fingers [13], not even of those in ING proteins.

Recently, it has been reported that the ING PHD fingers bind to H3K4me3 [9]. The binding site on ING2 has been mapped by NMR and mutagenesis, and the three-dimensional structure of the complex determined by crystallography [10]. We have confirmed by NMR that ING4 PHD finger binds to H3K4me3 peptide, and mapped the binding site (Figs. 2 and 3). The CSP measured in the presence of 1:4 excess of peptide is represented for each residue in Fig. 3. There are many residues that experience large perturbations in their chemical shifts, indicating a large interaction surface. There is a strong similarity with the pattern of changes experienced by ING2 PHD (see Fig. 2 of Ref. [10]), suggesting that the binding site is similar in both proteins. As shown in Fig. 3 (see also Supplementary Material Fig. 2) the PHD finger of ING4 binds to histone-3 and to its six possible methylated variants at K4 or K9 with the same binding site, but it does not bind to histone-4 or its different methylation states in K20 (only for H4K20me3 a few residues show changes just above the experimental error). The titration curves obtained for histone-3 peptides are shown in Fig. 4, and the calculated dissociation constants are summarized in Table 1. H3K4me3 peptide binds with a $K_D = 4.0 \pm 0.7$ μM, close to the value of 7.9 ± 2 μM measured previously [10] (by fluorescence, and possibly not exactly the same PHD chain length). Table 1 also contains the corresponding dissociation constants reported for ING2 PHD [10] for comparison. Both ING2 and ING4 bind to histone-3 methylated tails and not to histone-4, and bind to H3K4me3 with a similar affinity (considering the estimated errors). But beside these similarities there are remarkable differences. ING4

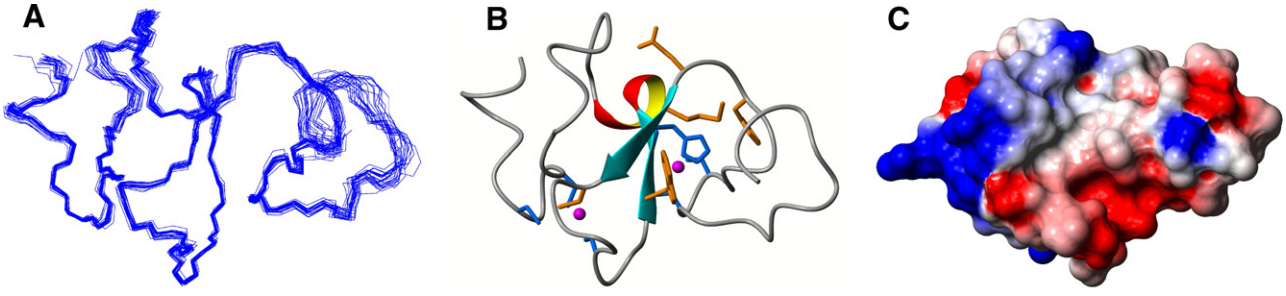

Fig. 1. Solution structure of the ING4 PHD finger: (A) ensemble of 25 refined structures; (B) ribbon model of one of the structures with the two Zn^{2+} ions in magenta, the side chains of the residues coordinating the ions in blue, and the side chains of the residues that experience the largest CSP upon binding to peptide H3K4me3 in orange (Y206, E208, M209, C212, W221, and G235 with CSP above the average plus one standard deviation) and (C) surface of the molecule coloured according to its electrostatic potential (negative in red and positive in blue). The three representations show the molecule in the same orientation.

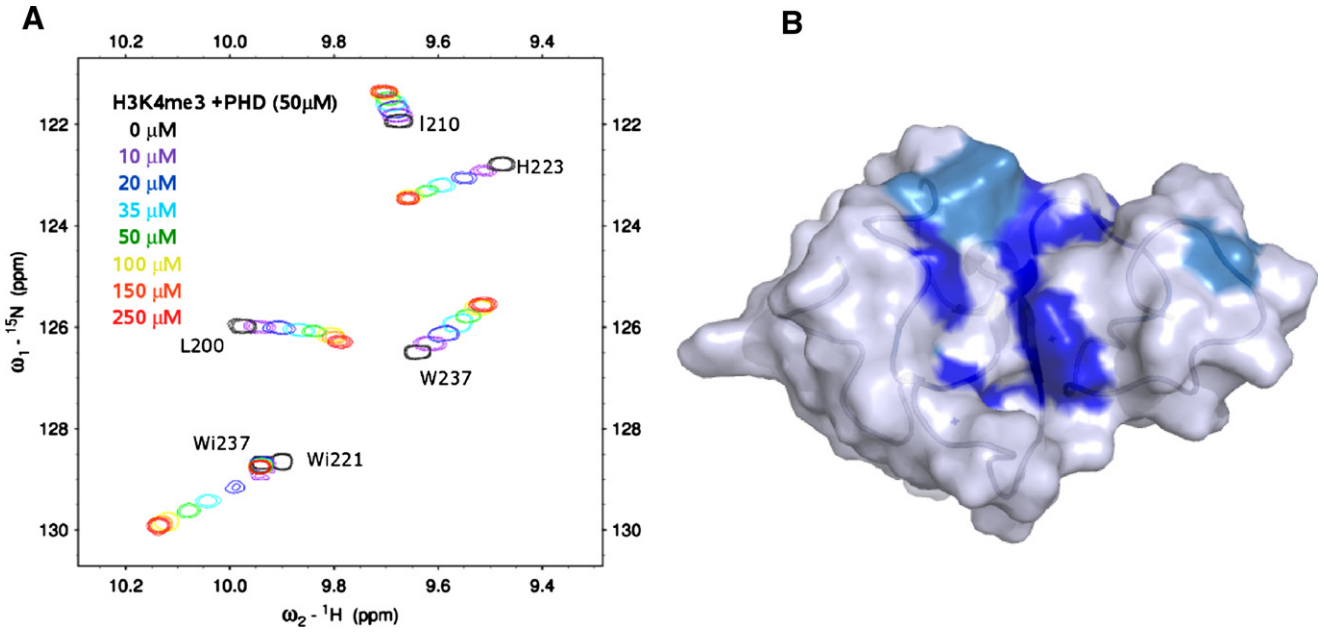

Fig. 2. (A) Superposition of a region of six ^{1}H–^{15}N-HSQC spectra of ING4 PHD after addition of different amounts of H3K4me3 peptide indicated with different colours. The labels adjacent to the signals indicate the corresponding residue ("Wi" stands for the triptophane indol NH signals). (B) Surface representation of the ING4 PHD finger (in pale blue with a ribbon model inside) with the same orientation as in Fig. 1. Those residues with a CSP upon binding to peptide H3K4me3 larger than the average plus one standard deviation (206, 208, 209, 212, 221, 235) are highlighted in dark blue, and those with a CSP larger than the average (192, 199, 204, 223, 232, 327) in light blue.

PHD is less selective than ING2 towards K4 versus K9 methylated H3. ING4 is also less selective towards the different methylation states of H3K4 than ING2. These measurements suggest a different mode of binding for the PHD. To better characterise the binding site and compare it to the mode of binding of ING2 we have analysed four alanine mutants of ING4 PHD. Y198A mutant corresponds to the Y215A mutation in ING2, which strongly decrease the affinity of ING2 for H3K4me3 [10]. The other three mutations map the relevance for binding of the residue that experiences the largest CSP (M209) and two other residues that are in regions where there is a cluster of large CSP (Fig. 3) and that were not probed in the ING2 study [10]: D192, at the N-terminal region, and W237, which lines a hydrophobic pocket where the methyl groups of the peptide A1 and T2 residues are buried. Mutant W237A is unfolded, as indicated by the sharp and non-dispersed signals observed in its NMR spectra (data not shown), and the addition of H3K4me3 was not enough to displace the folding equilibrium towards an NMR detectable population of the folded state. The other three mutants show dispersed ^{1}H–^{15}N-HSQC spectra and the dissociation constants were measured with a signal that was tentatively assigned to W237 according to its chemical shifts, similar to those measured for the wild-type (WT). As can be seen in Table 2, the three mutants bind to H3K4me3 peptide with affinities that are not very different from those of the WT (reduced or increased by a factor of 3 or 4). These results indicate that M209 plays a minor role in the binding even though is the residue that suffers the largest CSP, probably because it is very close to the trimethylated lysine, as occurs in the ING2 complex [10]. The D192 mutation is in the N-terminal region, and the methylated lysine points towards this end in the structure of the ING2 complex. This explains the observed CSP, and the affinity measured suggests that this region of the protein probably does not make a large contribution to the binding. This residue was not present in the shorter ING2 PHD [10]. Given the similar pattern of the CSP in both PHD fingers, we expected that mutant Y198A would show a very much reduced affinity for H3K4me3, as in ING2. On the contrary, the affinity is reduced but only by a factor of 4 while a three orders of magnitude reduction in the K_D was reported for ING2 PHD [10]. Although the explanation for this difference will only be possible after examination of the structure of the complex of ING4 PHD with the H3K4me3 peptide, this observation demonstrates that there are important differences in the mode of binding for the two molecules. This is consistent with the different selectivity towards the different methylation states of histone-3 discussed above, which is higher for ING2. In this respect, ING4 behaves as the WDR5 module of the MLL1 complex, which activates transcription via methylation of histone-3 [23]. WDR5 binds with similar affinity to the four H3K4 peptides (even the unmethylated one), and is proposed to present the K4 chain of histone-3 for further methylation rather than read its methylation state.

The binding of ING2 PHD to H3K4me3 has been related with transcriptional repression through the recruitment of ING2-HDAC1 complex at target promoters [9,10], and ING2 has been copurified with mSin3A deacetylation complex, which is also linked to repression [2]. However, the PHD finger of NURF helps to recruit the NURF remodelling complex to promoters and modulate transcription initiation [11]. The binding properties of the PHD finger of ING4 link it with actively transcribed genes, since trimethylation of histone-3 at K4 is a hallmark of active genes [24], while trimethylation in K9 (and in K20 of histone-4) is associated with gene silencing [25]. ING4 has been found to copurify with histone-4 acetyl transferase complex HBO1, involved in transcription activation [2]. Although the precise functional implications of

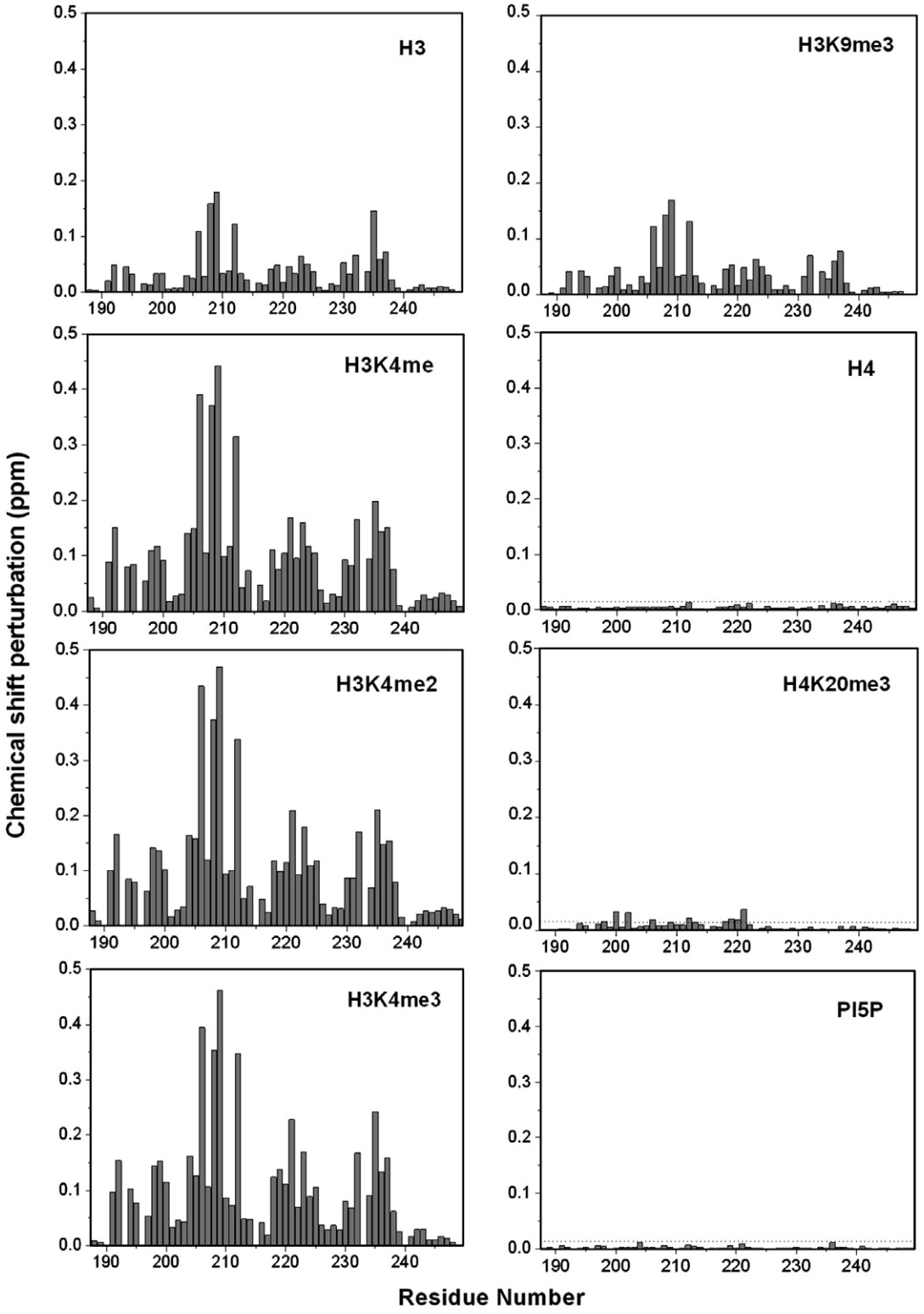

Fig. 3. Binding histograms showing the CSP observed for each residue in the ^{1}H–^{15}N-HSQC spectra of ING4 PHD in the presence of 1:4 excess peptide or 1:10 excess D-myo-phosphatidylinositol 5-phosphate (PIP5). The estimated experimental error (±0.012 ppm) is indicated by the dotted line in the two plots corresponding to peptide H4K20 peptides and PI5P binding.

H3K4me3 recognition by the PHD finger of ING4 are still to be determined, the results presented here show that it behaves differently from ING2. While ING2 PHD performs as a dual specificity module for both H3K4me3 and phosphatidylinositol-5-phosphate [9] and plays a role in transcription repression, ING4 PHD does not bind to phosphoinositides, binds to the three methylation states of H3K4 and is possibly involved in transcription activation.

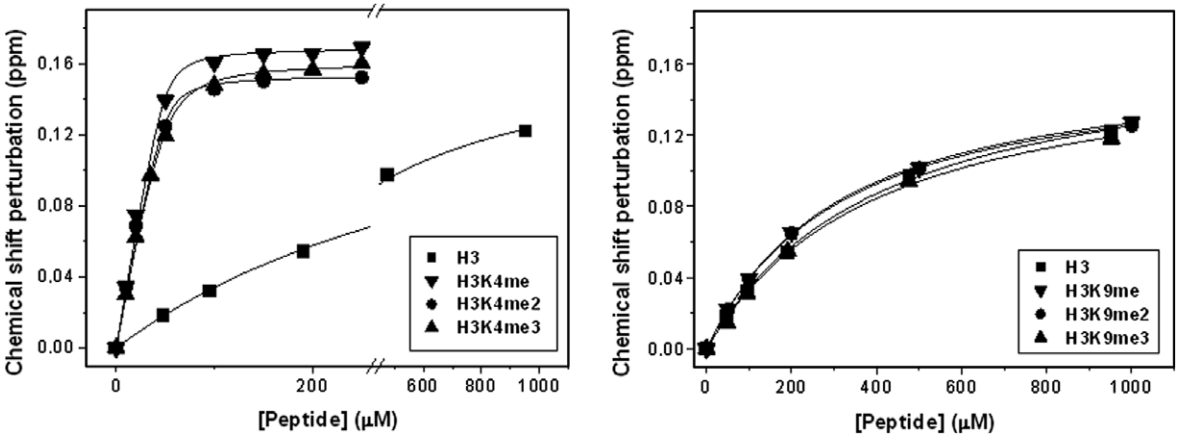

Fig. 4. Plots of the CSP of W237 amide resonance of PHD (50 μM) as a function of the concentration of H3K4 (left), or H3K9 (right) peptides. The symbol's height indicates the experimental error.

Table 1
Dissociation constants for the binding to histone peptides of the PHD fingers of ING4 (this work) and ING2 (Ref. [10])

Ligand	K_D ING4 (μM)	K_D ING2 (μM)
H3	370 ± 20	2240 ± 350
H3K4me	1.6 ± 0.8	208 ± 80
H3K4me2	1.8 ± 1	15 ± 4
H3K4me3	4.0 ± 0.7	1.5 ± 1
H3K9me	274 ± 8	2380 ± 800
H3K9me2	274 ± 6	2320 ± 300
H3K9me3	360 ± 30	2000 ± 60
H4	>10000[a]	n.d.[b]
H4K20me	>10000[a]	>7000
H4K20me2	>10000[a]	>10000
H4K20me3	7900 ± 200[c]	>10000

[a]When no interaction was detected we assume a lower limit of 10000 μM.
[b]Not determined.
[c]Data up to a 1:25 protein:peptide ratio were fitted assuming the same average $\Delta\delta_{max}$ obtained for the other peptides (0.16 ppm).

Table 2
Dissociation constants for the mutants of ING4 PHD (this work) and ING2 (Ref. [10])

Mutant	K_D ING4 (μM)	K_D ING2 (μM)
D192A	0.8 ± 0.6	n.d.[a]
Y198A	12.0 ± 0.8	>5000
M209A	17 ± 4	n.d.[a]
W237A	Unfolded	n.d.[a]

[a]Not determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.11.055.

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